Screening and Identification of LAB for Degradation of Nitrite in Sichuan Paocai Water

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Abstract. Paocai is a traditional fermented food, which is loved by people because of its delicious. However, nitrite inevitably exists in Paocai, and excessive intake of nitrite will cause a certain degree of harm to the human body. Studies have shown that LAB have the ability to degrade nitrite, and AD and ED are the two main ways. In this experiment, 20 LAB strains of that can degrade nitrite were screened from Paocai water. After 30 h, the NDR of DAE was 97.69%~99.71%. L2 and L6 could degrade nitrite by high efficiency ED, which were screened by spectrophotometer. The two bacteria's NDR of DAE was 99.28% and 99.13%, and the NDR of ED were respectively 90.89% and 90.02%. The two strains were identified by molecular biology. The results showed that L2 was Lactobacillus plantarum and L6 Lactococcus lactis.

Introduction

Nitrite is a common nitrogenous compound[1], which can cause poisoning after excessive intake[2]. It can be usually found in meat, vegetable, water and so on[3]. Paocai is a traditional fermented food with a long history in Sichuan, China. After being cleaned, vegetables are put into Paocai water for a certain time to ferment, thus forming a unique taste and flavor[4]. In the process of fermentation, LAB plays an important role. LAB can produce lactic acid in the process of fermentation to improve Paocai acidity and bring unique flavor[5]. Not only that, LAB plays a regulatory role in human intestinal health to promote the digestion and absorption of human intestinal flora[6]. However, Paocai still contains nitrite. With the emergence of multiple nitrate poisoning events[7], people pay more attention to nitrite in Paocai. Therefore, many scholars are concerned about reducing nitrite in Paocai.

Many researches have been proved that LAB could degrade nitrite[8], and some scholars have made mixed fermentation and achieved remarkable results. Some Paocai were found to contain less nitrite through researchers directly add LAB[9]. Zhou and others confirmed that inoculation of LAB fermentation can effectively reduce nitrite in food[10]. Kim have proved that most of the LAB have the function of degrading nitrite, those LAB degradation mechanism had been basically clear[11], ED at the early stage of fermentation, and AD at the later stage of fermentation[12].

At present, the NDR in the food field had become the hot problem[13]. Many studies had shown that LAB was beneficial to inhibit nitrite forming and reduce nitrite accumulation[14]. Therefore, this experiment used Paocai water as the material to screen LAB to degrade nitrite, meanwhile to stored bacteria resources for food industries.

Materials and methods

Sampling

Four home-made Paocai water were randomly sampled in Ya'an, sichuan province,which were kept at low temperatures and sent to the laboratory quickly.

Medium

MRS liquid medium: Peptone, glucose, potassium dihydrogen phosphate, yeast powder, diammonium citrate, sodium acetate, beef paste, manganese sulfate, TWAIN-80, magnesium sulfate, distilled water,

MA: MRS medium, 2% calcium carbonate, PH 6.4,

MSDN: MRS medium containing 125 mg/L sodium nitrite.

MSDNE: 125mg/L sodium nitrite, MRS medium with 2% calcium carbonate, PH 6.4.

Reagent

Glacial acetic acid, zinc acetate, nitrite, sodium borate, ferrous cyanide potassium, anhydrous ethanol (AR), p-phenyl sulfonic acid, naphthalene ethyl hydrochloride two amine, etc. All reagents were analytically pure.

Instrument and equipment

Super-clean worktable, Ultra-low temperature refrigerator, spectrophotometer, optical microscope, high pressure sterilization pot, constant temperature biochemical culture box, electric constant temperature blast drying box and so on.

Isolation and screening of LAB

Dilution of sample

The 25 mL sample was added to the 225 mL saline, and then 1 mL of which was added to 9 ml of saline. Repeat the operation until the sample is diluted to 10^{-8}.

Screening of LAB

$10^{-4} \sim 10^{-8}$ samples respectively were cultured by pouring in MRS medium at 37 ℃ for 36~48 h[15]. The calcium dissoluting zone of LAB colony needed to be observed after the culture[16]. Referring to the bacterial identification manual, the single colony of suspected LAB was picked out from the cultured samples, and was inoculated on the MRS medium for screening bacteria 37 ℃. After 48 h, single colony with marked calcium dissoluting zone were picked out for repeated culture to get LAB.

Characteristics and microscopic examination of colonies

The morphological characteristics of LAB were classified according to the manual of microbial identification[17].

Microscopic examination was observed with low magnification under ordinary optical microscope, and then using oil mirrors to observe the shape of LAB, which referred to the manual of microbial identification.

Screening and identification of LAB that degraded nitrite

Preliminary screening

Under the aseptic environment, the strains were activated in MA at 37 ℃ for 11~13 h. 10μL bacteria solution was added to 10 mL MSDN to be cultured at pH 6.4, 30℃, for 36~72 h.

The samples were used to react with naphthalene ethylenediamine hydrochloric acid (chromogenic reaction: 2 mL p-aminophenol sulfonic acid first, after 5 min, adding 1 mL naphthalene ethylenediamine solution, then observing and recording the color changes of each medium). Finally, the strains with lighter color were picked out as the strains screened preliminarily[18].

Re-screening

The strains came from preliminary screening was activated in MA for 12 h. 60 mL of cultured MA was transferred to MSDN(containing 125 mg/L $NaNO_2$ and 0.4 g $CaCO_3$), pH at about 6.4, then all the samples were cultured in shaking bed at 100 r/min, 37 ℃. The weight of $NaNO_2$ was conducted at intervals, and the NDR was calculated, judging by the spectrophotometer method in the chinese national standard *GB5009.33-2016*. At the same time, the standard curve and reagent blank were completed.

Reference to *GB5009.33-2016* for measuring the weight of nitrite, according to the absorbance of different concentrations of the standard $NaNO_2$ at 538 nm, draw the standard curve about A $_{NaNO2}$ $_{[L/(g \cdot cm)]} - C$ $_{NaNO2 \ (mg/kg)}$.

The weight of nitrite (based on sodium nitrite) is calculated by formula based on *GB5009.33-2016.*

Molecular sequence identification

Strains were identified using 16S rDNA gene sequencing. Omega's DNA extraction kit was used for total genomic DNA extraction, when strains grew to their late log phase. Fragments of bacterial

16S rDNA were amplified by polymerase chain reaction (PCR) using the primers 27F (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTACGACTT-3') (Bioengineering Co., Inc., Shanghai, China) with the thermocycler. PCR amplification products were sequenced by Sanger chain termination method and spliced by Contig Express splicing program. The reaction parameters of PCR included five min of denaturation at 95℃, followed by 35 cycles of 95℃,for 30 s,58℃, for 30 s, 72℃, for 90 s, and a final extension at 72℃, for 7 min. After the reaction, the PCR products were subjected to 1% agarose gel electrophoresis to confirm the PCR amplification fragments. The purified PCR products were sent to Shanghai Bioengineering Limited by Share Ltd to sequence. Finally, the spliced sequence file was compared with the data in the NCBI ribosomal DNA sequence (Bacteria and Archaea) database by using the NCBI Blast program, and the strain information with the largest similarity to the sequence of the tested bacteria was obtained. In order to further display the relationship and system status of the tested strains and the known LAB, according to the results of the homologous sequence search, the ClustalX software was used to match the multiple sequences of the tested strains and the related strains (Alignment), and the booststrap method and neighbour-joining methods in the MEGA 6 biological software were used to construct phylogenetic tree with 1000 Bootstrap tests.

Results and Analysis

Isolation and purification of LAB
40 strains with obvious calcium dissolving zone were isolated from Paocai water. 36 strains of Gram-positive bacteria were observed by Gram staining，and LAB were generally chained or paired without spores.

Preliminary screening of nitrite degrading strain
The 36 screened strains were activated in MA for 12~14 h, and then 10 µL bacterial fluid was transferred to MSDN to incubate at 30℃ for 42 h. Subsequently, after the chromogenic reaction, 20 strains with light color were selected out.

Rescreening of bacteria of degrading nitrite
The nitrite degradation rate was measured by spectrophotometer in GB 5009.33-2016，20 strains of bacteria were activated in MA , inoculated separately in MSDN and MSDNE，cultured at 30℃ in the shaking bed, the weight of $NaNO_2$ was detected after 6 h, 24 h and 36 h respectively, at the same time, reagent blank was set up to calculate nitrite degradation rate.

The degradation rate of nitrite
The nitrite degradation rate was measured according to the GB 5009.33-2016 spectrophotometer. The nitrite degradation rate of the 20 tested strains was obtained.

The results show that nitrite was firstly degraded by enzyme and then degraded by acid at the later stage of LAB fermentation. The NDR in all strains at 30 h could reach 97.69%，and the NDR of the 20 LAB strains was stronger.

The NDR by enzyme
These strains was inoculated in MRS medium containing $NaNO_2$ and $CaCO_3$, shaking culture at 30℃, 100 r/min, and the NDR of LAB were obtained.

Analyzing the data, we can conclude that at the early stage of LAB fermentation in Paocai water, the pH of the solution was about 6.4, and the nitrite was degraded through ED, which was degraded by nitrite reductase produced by LAB. It could be seen from table 1 and table 2 that when the 20 strains of tested bacteria were at a higher pH, their DNR decreased to some extent. However, it was obvious that the NDR of L_2 and L_6 could still reached more than 90% in a high pH , so L_2 and L_6 had strong ability of ED.

Identification of molecular sequence
The DNA of L_2 and L_6 were extracted by DNA extraction kit,which were amplified by bacterial 16S rDNA gene general primers to get 16S rDNA product. The results of agarose gel electrophoresis were Figure 1. The size of the product is about 1500 BP, which was consistent with the standard material, indicating that the amplification is successful[19]. The PCR products were

sent to Shanghai biological Co., Ltd. For sequencing, and the sequenced results were appended at the end of the article. The homology was compared with the sequence in Genbank, in the end, L2 was identified as *Lactobacillus plantarum* and L_6 *Lactococcus lactis*. C1ustalX software was used to analyze multiple sequences of test strains and related strains (Alignment). The phylogenetic tree was constructed by using booststrap method and neighbour-joining methods in MEGA 6 biological software, and 1000 Bootstrap tests were performed, and the result was showed in Figure 2. L2 and L6 were clustered together, and their genetic relationships were very close, but they were far from *Lactococcus lactis subspecies AB100803.*1, *Lactobacillus plantarum Argentina subspecies AJ640078.1, Lactobacillus plantarum KX057547.1, Wechsler AB023236.1 and AF111948.1.*
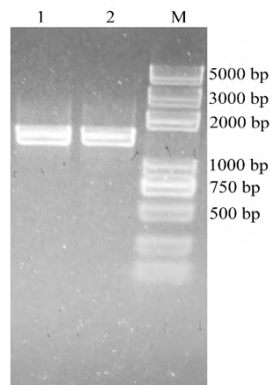

Figure 1 16S rDNA agarose gel electrophoresis of L2 and L6
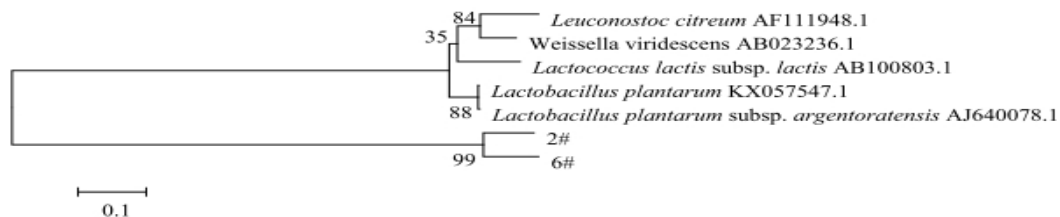M：DL 1500 DNA Marker,   1: L_2 , 16S rDNA,   2: L_6, 16S rDNA

Figure 2 Phylogenetic tree of L_2 and L_6

Discussion and Conclusion

Discussion
Time for nitrite reaction with LAB
According to the previous research, the experiment was conducted at 6 h, 24 h and 36 h asthe time point for measuring the degradation effect of nitrite.

The results of the pre experiment showed that the degradation rate of nitrite was little changed after 30 h, and Hai Qing et al[20]directly detected the NDR after 24 h. So in this experiment, the degradation rate was determined after 30 h.[21]

The ability of LAB to degrade nitrite in 6~24 h was not guaranteed to be the strongest. Therefore, the test of the NDR of 6 h, 24 h and 30 h was suitable for this experiment.

The results showed that, whether DOE or DOA, the NRD was not high after 6 h. It was probably due to the low acidity at the early stage of fermentation, mainly depending on the DOE of LAB[22], and the growth of LAB was in the delay period, so the effect of the DON was not obvious.

Methods for screening bacteria
In this experiment, the preliminary screening was carried out first and then the rescreening, which was based on the experience of many scholars before.

Facts showed that bacteria with a weak ability to degrade nitrite could be largely removed by preliminary screening. But it could not be accurate, and the error was larger because it was observed by the naked eyes.

The rescreening could   improve the accuracy of the measurement. In addition, preliminary

screening could do better to relieve the work of rescreening. So preliminary screening firstly and then rescreening could not only ensure the accuracy of the results, but also improve the detection efficiency[23].

Conclusion

In China, the annual output of vegetables is high, but the preservation of vegetables has not been effectively solved, so many people make vegetables into Paocai water , and Paocai has a lot of beneficial effects on the human body and is widely favored by people. However, the excessive nitrite in Paocai has always been a hot issue. So in this experiment, 20 strains of LAB capable of degrading nitrite were screened out in Paocai water.

40 strains with the ability of dissolving calcium were isolated and purified from Paocai water, of which 36 were Gram-positive bacteria, and 20 strains of LAB that could degrade nitrite were screened at the end of preliminary screening.

DOE in the early stage of fermentation, DOA in later period, LAB degraded nitrite through the effect of nitrite reductase, pH 6.0~6.4, compared with nitrite degradation ability in the ordinary screening medium without acidity control, 20 strains found in the preliminary screening could degrade nitrite obviously during the fermentation process. After 30 h fermentation, the NDR can reach 97%.

When the pH was controlled between 6.0~6.4, the LAB degraded nitrite through DOE, and the NDR of the 20 strains generally decreased, but the L_2 and L_6 still had high NDR. The NDR of L_2 reached 99.28% after 30 h, the NDR by enzyme was 90.89%. The NDR by enzyme of L_6 was 90.02%. Therefore, it could be concluded that nitrite reductase of L_2 and L_6 had obvious effect and enzyme degradation ability was better than other strains.

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Abbreviation in this article

LAB: lactic acid bacteria, AD: acid degradation, ED: enzyme degradation, NDR: nitrite degradation rate, DAE: degradation of acid and enzyme, MRS: De'Man Rogosa Sharpe, MA: medium for activating strains, MSDN: medium for screening strains that degrade nitrite, MSDNE: medium for screening strains that degrade nitrite by enzyme